HHS Public Access

Author manuscript

Curr Opin Struct Biol. Author manuscript; available in PMC 2017 October 13.

Published in final edited form as:

Curr Opin Struct Biol. 2016 October ; 40: 145–152. doi:10.1016/j.sbi.2016.09.013.

Multi-level regulation of cellular glycosylation: From genes to transcript to enzyme to structure

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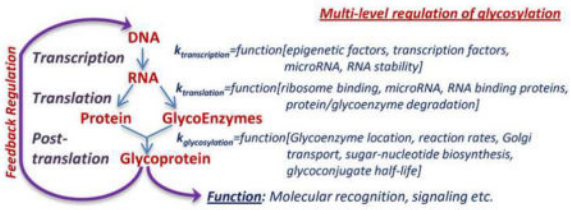
Abstract

Glycosylation is a ubiquitous mammalian post-translational modification that both decorates a majority of expressed proteins and regulates their function. Cellular glycan biosynthesis is facilitated by a few hundred enzymes that are collectively termed ‘glycoenzymes’. The expression and activity of these enzymes is controlled at the transcription, translation and post-translation levels. New wet-lab advances are providing analytical methods to collect large-scale data at these multiple levels, relational databases are starting to collate these results, and computer models are beginning to integrate this information across scales in order to gain new knowledge. These activities are likely to enable the qualitative and quantitative mapping of pathways regulating glycan production and function in proteins, cells and tissue.

Graphical Abstract



Introduction

Cell surface, secreted and intracellular proteins and lipids are extensively glycosylated. These modifications affect a range of molecular recognition and signaling events [1]. Whereas intracellular glycoproteins often contain short carbohydrate structures, commonly bearing a single O-linked GlcNAc (N-Acetyl glucosamine) residue, extracellular glycans

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bear more elaborate structures [2]. On human cells, these include branched O- and N-linked glycans, glycolipids and repeating glycosaminoglycans (Figure 1). These sugars are commonly made up of nine different monosaccharides. Altogether, human glycoproteins and glycolipids are estimated to contain a minimum of 3,000 unique 2–5 monosaccharide determinants [3]. Individual cell and tissue only have a subset of the glycoenzymes and thus individual glycome typically have fewer members. Similarly, glycosaminoglycans are estimated to contain up to ~4,000 possible pentasaccharide sequences [3]. These determinants provide the recognition motifs underlying the biological functions of glycans.

Classical studies into the biosynthesis of glycan epitopes use reductionist, biochemical methods to elaborate individual biosynthetic steps. In recent years, however, newer ‘omics’ approaches are rapidly bridging gaps in our knowledge of glycan biosynthesis, especially with respect to cell-type and context-specific glycosylation [4–7]. These newer experimental modalities often use mass spectrometry and lectin microarray based technologies [8]. Integrating these glycomics data sets with high-throughput genomics, transcriptomics, enzymology and proteomics methods is necessary as it can lead to a ‘systems level’ understanding of glycan biosynthetic pathways [9]. This review describes progress and challenges in the development of such systems-based understanding, with emphasis on computer based modeling and pathways regulating canonical O- and N-linked glycosylation in humans.

Multi-level regulation of glycosylation

The regulation of glycoproteoforms (i.e. the glycosylation variants of a protein) in metazoans is complex. It involves not only the biosynthesis of the underlying protein scaffold built on its mRNA template, but also non-template driven post-translational modifications that is driven by the glycosylation-related enzymes or ‘glycoenzymes’. In humans, ~500 glycoenzymes participate in this process including 209 glycosyltransferases, 76 glycosidases, 114 enzymes involved in sugar metabolism & transport, 54 sulfation-related enzymes and 31 enzymes regulating additional lipid and GPI-anchor biosynthesis pathways [10,11]. Regulation of protein scaffolds and glycoenzymes (themselves proteins) underlying glycoproteoforms occurs at multiple steps, including transcriptional, translational and post-translational levels (Figure 2).

Transcription is perhaps the most recognized regulator of protein, and thus glycoproteoform, expression. Transcription of a glycosylation-related gene (glycogene) is controlled by both transcription factors binding to gene promoter and enhancer elements, and by epigenetic regulation of gene accessibility [12]. Transcription factors, which can act as promoters or repressors, are often glycosylated by O-GlcNAc which can regulate the transcriptional machinery [2]. In general, there is little available information about transcription factor-mediated control of glycogene expression, with only ~2 dozen glycogene-transcription factor interactions known to date and mechanistic work available for ~15 glycogenes [13–16]. The importance of transcription factors in control of glycosylation is exemplified by recent work on the transcription factor XBP1s, a major regulator of the unfolded protein response (UPR) [15]. Here, the artificial activation of XBP1s induced expression of a series of *N*-glycan biosynthetic enzymes, shifting the glycosylation profile of a secreted

glycoprotein from oligomannose towards more processed hybrid and complex epitopes, tying together the UPR with changes in the glycome [15]. In another example, the phosphorylated form of the transcription factor ATF2, which has a role in therapeutic resistant melanoma, was recently found to directly repress expression of fucokinase (FUK). This enzyme is a key component of the fucose salvage pathway and its repression by activated ATF2 resulted in ~40% loss of cellular fucosylation [16]. Beyond transcription factors, access to the genome, which is controlled by epigenetic modifications such as acetylation, methylation and ubiquitinylation also regulate glycogene transcription [12]. This is even less studied than transcription factor binding to glycogenes, although a recent study analyzing DNA methylation of 86 glycogenes suggests a positive correlation between the promoter methylation status of glycogenes and expected tumor-associated glycan structures [17]. A handful of studies have also shown a direct interaction between the methylation status of specific glycogenes and glycogene expression [18–21]. In this regard, the methylation of the α(2,3)sialyltransferase ST6Gal1 promoter was shown to silence expression of the ST6Gal1 transcript in bladder cancer [18], confirming an earlier report in breast cancer showing methylation-dependent silencing of this glycogene [22]. Mapping transcription factors and epigenetic regulation onto glycogene regulatory networks will require a combination of methods from traditional Chromatin Immunoprecipitation (ChIP) assays to more sophisticated promoter-reporter assays that can reveal indirect and direct effects, to more sophisticated analysis of genome accessibility and silencing [15,23]. All of this is important to our understanding of the glycome and its regulation.

Although transcript (i.e. cellular mRNA) levels are often used as a key indicator of what glycogenes are being translated and the levels of biosynthetic enzymes and glycans that result, the relationship is not straightforward. A number of investigators have attempted to relate transcript levels to glycoenzyme activity and carbohydrate structures, including the response of the system to perturbations [11,24–26]. Such studies have been conducted using human promyelocytic leukocytes differentiated to primary neutrophils [24], a limited panel of mouse tissue [11], mouse embryonic stem cells differentiated to embryoid bodies and endodermal cells [25], and epithelial ovarian cancer cell types [26]. The broad conclusion of these studies is that glycogene, and more specifically glycosyltransferase, expression measurements in a number of cases can semi-quantitatively predict corresponding enzyme activity and cell-surface glycan expression. However, the relationship is often non-linear and in some instances, changes in glycan structures upon perturbation do not correlate with changes in glycogene mRNA expression levels. Although part of this discrepancy may be due to difficulties with quantitatively measuring low-abundance glycogene transcript levels [11], the post-transcriptional regulation of glycogene expression may also control carbohydrate structures.

MicroRNAs (miRNAs), small non-coding RNAs, are major post-transcriptional regulators of protein expression, acting as rheostats to maintain key proteins critical to a cell state at set levels [27,28]. Genes with lower expression levels, such as glycogenes, are both preferentially targeted by miRNA and more impacted by miRNA binding [28]. Recent work has shown that miRNA are indeed a major regulator of the human glycome and the impact of miRNA on the glycome via alteration of glycosyltransferase protein levels may explain discrepancies with the transcriptome [4,29]. For example, miR-34a, a microRNA associated

with hepatocellular carcinoma, targets FUT8. This miRNA did no impact FUT8 transcripts in human HepG2 cells, although it concomitantly altered FUT8 protein expression and core fucosylation levels [30]. miRNA regulate biological pathways by regulating sets of gene transcripts. Multiple laboratories have demonstrated that downregulation of single glycogene (or other gene) targets of miRNA mimic the biological effects of the miRNA [31–33]. Thus, miRNAs could be used as a proxy to identify glycosylation enzymes involved in the pathways they regulate (miRNA proxy approach) [32,33]. The miRNA proxy approach has been applied to identify functionally relevant glycogenes impacting cell states like epithelial-to-mesenchymal transition (EMT) [32] and the cell cycle [33]. For example, miR-200b, a regulator of EMT, was found to regulate a set of glycogenes from hard to analyze cohorts those involved in glycolipid (ST3GAL5 and ST6GALNAC5) and non-canonical O-glycan biosynthesis (B3GLCT). Downregulation of these glycogenes using shRNA phenocopied the miRNA, showing a role for these genes in the control of EMT [32]. This work opens up the possibility of leveraging post-transcriptional regulation of glycosylation as a means to reveal critical functional changes in the glycome that underlie biological processes. This may be especially important for the portions of the glycome, such as glycosylaminoglycans and glycolipids, which are overlooked in most analysis due to analytical challenges.

In addition to transcription, the measurement of glycoenzyme translation, turnover rates and activity are key parameters controlling glycan biosynthesis. In this regard, newer technologies like ribosome profiling are enabling a more nuanced understanding of protein synthesis [31]. This technology uses deep sequencing to identify stretches of mRNA that are actively bound to the ribosome during *in vivo* translation. Such quantitative estimation of protein biosynthesis rates may provide a new avenue for evaluating translation, not just transcription, as a measure of glycoenzyme expression, taking into account posttranscriptional regulation by miRNA.

Beyond expression, the direct measurement of glycoenzyme activity *in situ* is important. However, currently there is no established method for this measurement in the milieu of the Golgi. In complex mixtures, enzyme activity is typically followed in reaction vessels by assaying the conversion of a synthetic acceptor to product using cell/tissue lysates as the enzyme source. Product quantitation methods range from radioactivity based chromatography assays [34], to fluorescence based strategies that quantify glycan biosynthesis in an array format [35,36] and mass spectrometry [37]. Here, solution based enzymology assays are more sensitive compared to studies performed in 2D-array format, since only limited amounts of the carbohydrate acceptor can be immobilized on an array spot. As an alternative, mass spectrometry can be used to follow glycan biosynthesis in cells by feeding them small molecule acceptors, such as per-acetylated Benzyl-α-GalNAc, and subsequently measuring carbohydrate products formed on the synthetic substrate [38]. Data from such experiments, in tandem with genome editing methods (TALEN, CRISPR-Cas9 etc.), may enable estimation of glycosyltransferase activities and dissection of the interdependencies among glycogenes in the same biosynthetic pathway [5,39].

Overall, high-throughput analysis of the transcriptome is starting to provide clues to the nature of the cellular glycome and its response to system perturbation. Integration of

additional data including miRNA, glycoenzyme expression and activity is necessary in order to obtain a more complete picture of the glycan regulatory process.

Integrating knowledge using computer models of glycosylation reaction networks

The analysis of large-scale experimental datasets available from the above technologies may be streamlined by developing computational tools that can integrate results collected using these diverse methods. While such computational tools have been widely developed for genomics and proteomics research, they lag behind in the glycosciences [9,40]. This is in part due to the greater complexity of this research field, since glycoconjugate biosynthesis is regulated by multiple pathways including: i. sugar-nucleotide donor biosynthesis (e.g. UDP-Galactose) in the cellular cytoplasmic and nuclear compartments; ii. glycosylation reactions in the endoplasmic reticulum (ER) and Golgi cisternae; iii. transport processes that result in the sub-cellular localization of glycoenzymes, glycoconjugates and other reaction components in specific compartments; iv. trafficking of glycoproteins and lipids through the ER and Golgi which is impacted by cellular lectins and other transport mechanisms; and v. salvage pathways that aid the recycling of glycoconjugates back to their basic building blocks (Figure 2).

Integration of data across scales requires a robust modeling framework to formulate glycosylation reaction networks, integrate data from different relational databases, and enable code sharing among laboratories. Due to the last requirement, the Systems Biology Markup Language provides the ideal schema for model building [41,42]. Although this modeling language does not have specific facilities to handle glycan structures and glycosylation related reaction pathways, the annotation fields of the eXtensible Markup Language (XML) files can be easily modified to store pertinent information. For example, the annotation element of the *Species* field could store glycan structure information in XML format, the *Reaction* field can hold glycoenzyme and carbohydrate transport definitions/data, and *Compartment* is available to describe the physical features and reaction mechanisms in the ER and Golgi cisternae [42,43].

Some initial examples of glycosylation pathway modeling have appeared, and these investigations suggest that quantitative analysis of glycan structure data using computer modeling can yield novel biological insight. Notably, the computer models of Lau *et al.* showed that the activities of various N-acetylglucosaminyltransferase enzymes (MGAT1–5) together with the intracellular concentrations of UDP-GlcNAc regulate the pattern of N-glycan branching on cell surface proteins, especially growth factor receptors [44]. Higher levels of N-glycan-branching results in more robust receptor cross-linking (or lattice formation) using multivalent galectin-3, longer receptor retention/lifetimes on the cell-surface, and consequently more pronounced cell signaling. In another study of O-linked glycosylation, Liu *et al.* studied the critical enzymes (fucosyltransferases and sialyltransferases) regulating microheterogeneity in the leukocyte cell surface protein P-selectin glycoprotein ligand-1 and leukocyte cell adhesion function [45]. Predictions from such modeling have been validated by developing RNA-interference and CRISPR-Cas9

based knockouts [39,46]. In another example, Bennun *et al*. modeled cancer cell transcriptome and glycome data to demonstrate augmented α(1,2)fucosylation on prostate cancer cells during their transition from androgen-dependent to androgen-independent lymph node carcinoma [47]. Besides these studies that are of biomedical relevance, several studies have modeled the glycosylation status of recombinant proteins produced using CHO cell lines as this is of biopharmaceutical relevance (reviewed in [48]).

While some examples of glycosylation networks have appeared as illustrated above, additional development of machine-readable glycoenzyme definitions can accelerate such modeling efforts as this will enable: i) model-sharing among investigators, and ii) streamlined *in silico* description of different biological aspects such as sugar-nucleotide biosynthesis, species transport and rate expressions. In this regard, XML-based definitions for glycosyltransferases and glycosidases have appeared for O- and N-glycosylation pathways [42,43]. Succinct, computationally efficient definitions for the specificity of enzymes involved in N-linked glycosylation have also been described, though these definitions appear in string rather than XML format [47]. McDonald *et al*. also describe a similar modeling language specifically for O-glycosylation [49]. It seems possible that the merits of the above approaches may be combined to develop a more universally acceptable and comprehensive glycoenzyme database. Besides being linked to the GenBank for gene-level information and the carbohydrate-enzyme database CAZY for glycoenzyme structure and carbohydrate-binding module information [50], such a resource may also be parameterized using enzyme catalytic activity and rate constant data stored in BRENDA. Additionally, the glycoenzyme definitions may include reaction descriptions from the IUBMB ExploreEnz database, and pathway data from KEGG GLYCAN, an extension of the Kyoto Encylopedia of Genes and Genomes (KEGG) [51] (Table 1). Such efforts can enable the seamless integration of computational models with a variety of glycosylation related databases.

In addition to model formulation, advanced computer simulation methods are also needed in the field. In this regard, glycosylation reaction networks typically have a large number of reactions and reactants, but relatively few model parameters due to the limited number of glycoenzymes in a given system. Fitting such over-determined systems using deterministic ordinary differential equation (ODE) networks is complicated, though this is commonly done [44,45,47,52]. In such studies, optimization approaches like genetic algorithms are sometimes used to determine the *bounds* of the enzyme rate constants rather than exact solutions [45]. Another approach uses stochastic Markov chain methods for data fitting [53]. This method is computationally efficient but empirical (i.e. not mechanism based) in that similar reactions catalyzed by the same enzyme may have different rate constants. Besides these approaches, there are few examples of other computational techniques in the field like Boolean networks, agent-based models and statistical modeling [40]. Overall, streamlined methods to formulate and solve glycosylation-related mathematical models are awaited.

Besides data collected by individual laboratories, relational databases with systems-level information necessary for mathematical modeling of the glycome are starting to emerge (Table 1). Some of these repositories are a results of efforts by large consortia supported in the USA (Consortium of Functional Glycomics [CFG], www.functionalglycomics.org) [54],

Europe (EuroCarbDB) [55] and Japan (Japan Consortium for Glycobiology and Glycotechnology Database [JCGGDB]). Among these, at the transcript level, the CFG had developed glycogene specific microarrays and deposited related experimental data for two model organisms (human and mouse) under a limited range of experimental conditions [54]. These entries complement more generic transcriptomics data stored in the GEO (Gene Expression Omnibus) database. The JCGGDB also maintains a glycosylation-related disease database, which is an abridged form of the OMIM (Online Mendelian Inheritance in Man) catalog. MALDI-TOF mass spectrometry experiments that profile the N- and O-glycome of cells and tissue by analyzing the distribution of carbohydrates released from their underlying scaffolds are stored at the CFG database. The Unicarb-KB database catalogs intact glycoproteomics analysis mostly from literature [56]. While there is no specific database for the storage of lectin array results, the CFG has developed glycan microarrays that complement these efforts. Here, the binding specificity and affinity of glycan binding proteins (lectins) and antibodies to carbohydrates printed on chips is provided [4,57]. Complementing this, the JCGGDB has mapped the binding specificity of a range of glycan related antibodies (Table 1). The application of computational modeling concepts to quantitatively analyze genomics and proteomics resources stored in these databases is an important future step for our understanding of glycosylation processes.

Conclusions and outlook

The review highlights the manner in which glycans are synthesized and the myriad of control mechanisms regulating this process. The development of computational models to relate data available from different experimental modalities can provide a platform, initially for the qualitative understanding of these glycosylation reaction networks and subsequently for providing quantitative testable hypotheses.

Acknowledgments

SN is supported by NIH/NHLBI grants HL103411 and the Program for Excellence in Glycosciences award HL107146. LKM is supported by the NIH/NIAID grant U01AI111598.

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HIGHLIGHTS

- Glycosylation is regulated at the transcriptional, translational and post-translational levels
- Advances in sequencing and structural analysis enable data collection at all levels
- Relational databases that store glycosylation-related information are starting to emerge
- Computational models can integrate knowledge across different experimental scales

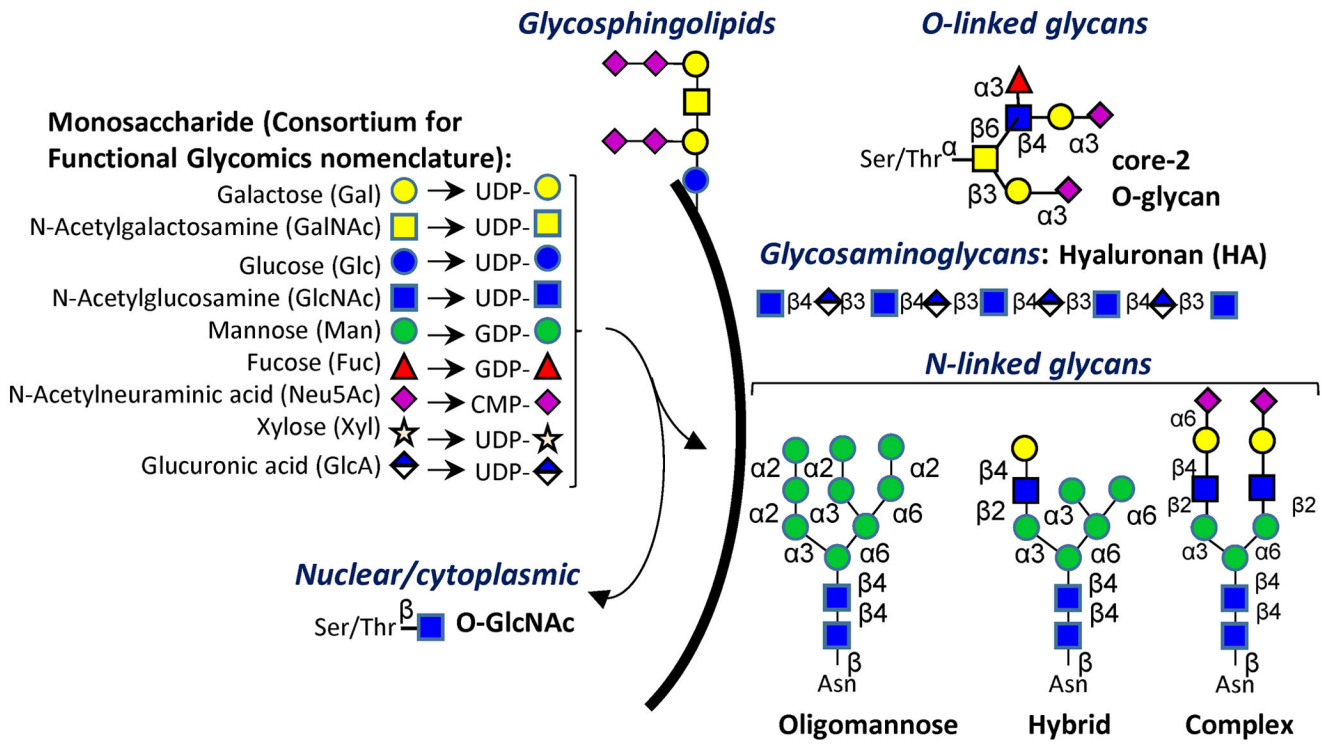

Figure 1. Glycosylation overview

Commonly, nine types of monosaccharides in humans form corresponding sugar-nucleotides (UDP-Gal etc.). A variety of glycoenzymes (particularly the glycosyltransferases and glycosidases) enable the transfer of these monosaccharides to lipid and protein scaffolds. Additional monosaccharides can form, e.g. Iduronoic acid (IdoA), by the epimerization of Glucuronic acid (GlcA) on glycosaminoglycan chains. Carbohydrate structures thus formed decorate the cell surface, and they are also found in intra-cellular compartments. Most glycan families have a limited number of core-structures that are elaborated by repeat extensions (like N-Acetyllactosamine repeats) and terminal modifications.

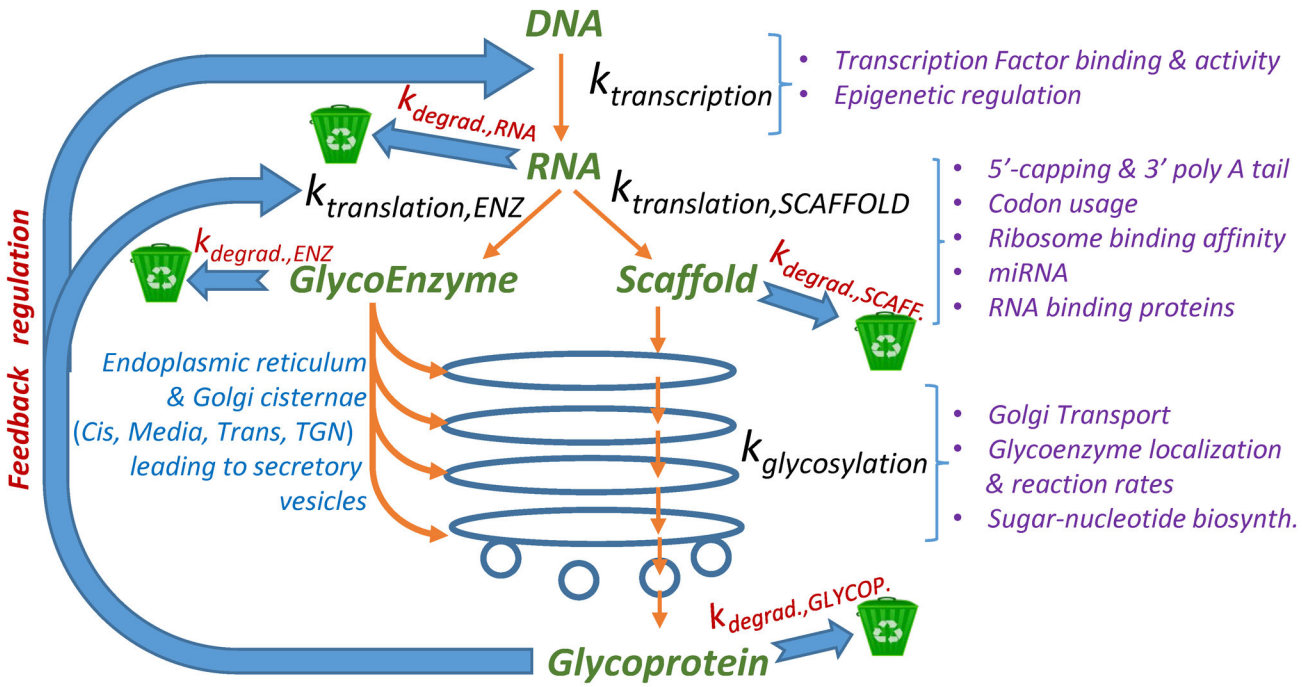

Figure 2. Regulation of glycosylation

The central dogma of molecular biology states the passage of information from DNA to RNA to protein in all living organisms. These proteins can be further glycosylated by the glycoenzymes, mainly in the endoplasmic reticulum and Golgi compartments. RNA and protein degradation rates, shown using recycle bins, are important checkpoints that control cell surface carbohydrate structures. Additional parameters that track the process of information transfer from genes to RNA to proteins (including glycoenzymes) to glycoproteins are shown using purple text. Glycoproteins may provide feedback and feedforward control to regulate cellular transcription and translation. The parameter '*k*' (e.g. $k_{transcription}$ and $k_{degrad.}$) is used to denote lumped rate constants for individual steps.

Table I

Databases for the Glycosciences *

Level	Description and unique feature	Website
Glycan entries	GlyTouCan	glytoucan.org
	UnicarbKB	unicarbkb.org
Enzyme data	CAZY (Carbohydrate-Active Enzyme)	www.cazy.org
	IUBMB ExploreEnz database	enzyme-database.org
	BRENDA (Comoprehensive enzyme info. system)	brenda-enzymes.org
Transcript	Glycogene microarray data - for human/mouse samples (CFG)	www.functionalglycomics.org
	Gene Expression Omnibus (GEO)- functional genomics repository	www.ncbi.nlm.nih.gov/geo
Glycomics/Glycoproteomics	GlycoBase	glycobase.nibrt.ie
	UniCarb-KB	www.unicarbkb.org
	CFG MALDI-TOF/TOF datasets	www.functionalglycomics.org
Glycan binding data	CFG glycan array entries	www.functionalglycomics.org
	LfDB (Lectin Frontier Database) & GlycoEpitope	jcggdb.jp/index_en.html
Pathway	KEGG GLYCAN	www.genome.jp/kegg/glycan

* This table only lists relational databases that would be useful for computational modeling of cellular glycosylation biosynthetic pathways. For a more comprehensive list of all glycan-related databases, please see ref. [9].